A Sensitive Assay for Xanthine Oxidase Using Commercially Available [^{14}C]Xanthine

A scaled-down radiochemical assay for xanthine oxidase using commercially available [^{14}C]xanthine is described. The substrate is purified by polyvinylpyrollidone column chromatography to decrease blank values. Inhibitors, if present, are removed from blood serum by gel filtration. Products are eluted from Dowex 50W and quantitated by liquid scintillation counting. Linearity was observed from 0.01 to 36.4 μU/sample.

Xanthine oxidase (XO; EC 1.2.3.2), which catalyzes the oxidation of a wide range of substrates, including xanthine, may be quantitatively determined by several different methods (1–8). Because of the current interest in the physiological role of XO (9), there is a need for a sensitive, convenient assay for the enzyme, e.g., to facilitate studies of intestinal absorption of bovine milk XO so as to be able to determine whether the enzyme finds its way into the blood stream and aortal and myocardial tissues. Ramboer (6) and Al-Khalidi *et al.* (7) developed sensitive assays for measuring the minute amounts of enzyme found in blood serum. This report describes a more sensitive assay adapted from these two methods.

The procedure consists of purification of commercially available [^{14}C]xanthine, gel filtration of xanthine oxidase samples containing low molecular weight inhibitors, incubation of enzyme with [^{14}C]xanthine, and scintillation counting of products recovered from Dowex 50W.[1]

MATERIALS AND METHODS

Poly-*N*-vinylpyrollidone (PVP) (Polyclar AT) was obtained from GAF Corp. The PVP was washed with water and the fines were decanted. The [8-^{14}C]xanthine (specific activity, approximately 62.8 mCi/mmol) was purchased from ICN Pharmaceuticals, Inc., and the xanthine (Sigma grade) and xanthine oxidase (Grade 1, from buttermilk) were from Sigma.

Purification of [^{14}C]*xanthine.* A PVP column (0.5 × 7 cm) was prepared using a Pasteur pipet, and the PVP was equilibrated with 0.07 M phosphate

[1] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

buffer, 1 mM EDTA, pH 8.3. A 0.095-ml aliquot of the [^{14}C]xanthine solution (0.151 μmol) was applied to the PVP column and eluted with the phosphate buffer. The first 2.3 ml of eluate was discarded. The purified substrate eluted in the next 1.8 ml was collected and stored at 4°C for use in the assay. It remained stable for at least 4 days. The purified substrate can be autoclaved at 121°C for 15 min to prevent bacterial decomposition of xanthine (7), which may occur with storage.

Sample gel filtration. Samples which may contain low molecular weight inhibitors should be filtered through Sephadex G-25 (fine) prior to assay (6). Pig blood serum (miniature, Hormel strain) was stored at −20°C, and enzyme activity was determined within 8 hr after collection. Aliquots of 0.3 ml serum were eluted from a Sephadex G-25 Pasteur pipet column (0.5 × 9.5 cm) with 1% bovine albumin in 0.9% NaCl. The initial 1.0 ml of eluate was discarded, and the next 1.2 ml, which contained the total protein, was assayed immediately.

In order to prepare enzyme standards for use in the radioactive assay, commercial buttermilk XO was assayed spectrophotometrically (8,10) to confirm the designated activity. A 1-ml aliquot of xanthine (11.4 mg/500 ml) was incubated with 1.9 ml of 0.07 M phosphate buffer (1 mM EDTA, pH 8.3) and 0.1 ml of enzyme (diluted with buffer to contain 0.1 to 0.2 units/ml) for 5 min at 25°C. The reaction was terminated with 0.5 ml of 40% trichloroacetic acid (TCA) (w/v). A unit of XO activity was defined as the amount of enzyme which catalyzed the formation of 1 μmol of uric acid/min at 25°C. The molar absorbance of uric acid at the pH of the solution is 1.16×10^4 cm^{-1}.

Standards for radioactive assay were then prepared by diluting the XO with 1% bovine albumin (in 0.9% NaCl) and assayed with and without Sephadex filtration. Buttermilk xanthine oxidase was also diluted (1:10) with fresh miniature pig serum and filtered through Sephadex, and the enzyme activity was determined (Fig. 1).

Radiochemical enzyme assay. A 0.15-ml aliquot of the purified [^{14}C]-xanthine (0.012 μmol) was incubated with each 0.2-ml sample at 37°C for 3 hr in 12 × 75-mm culture tubes covered with Parafilm. After addition of 0.05 ml 40% TCA (w/v), the mixture was centrifuged and the supernatant was removed. Samples which contained greater than 6.5 μU of xanthine oxidase were heated in boiling water for 10 min and cooled to room temperature. The increase in counts found following heating may be attributed to solubilizing the products. A 0.10-ml aliquot of supernatant was applied to a 5-cm Pasteur pipet column of Dowex 50W-X8-H$^+$ (200–400 mesh), which had been washed with 0.1 N HCl. The initial 0.6 ml of 0.1 N HCl eluate was discarded, then 1.3 ml was collected in a vial and counted in a liquid scintillator counter (Nuclear Chicago, Mark I) with 15 ml of Aquasol (NEN). For samples containing greater than 3.2 μU of XO, it was necessary to count an addi-

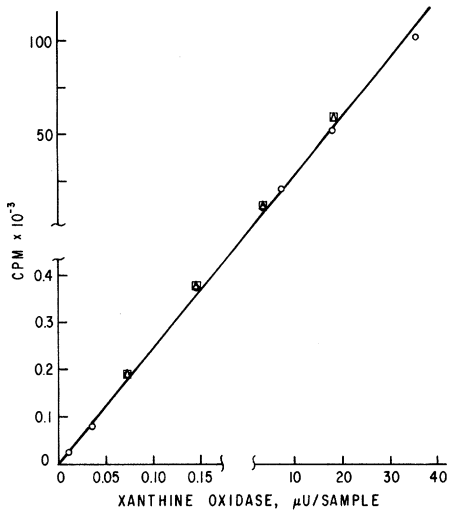

FIG. 1. Linearity of the radioactive assay for products from [^{14}C]xanthine with increasing concentration of buttermilk xanthine oxidase (diluted with albumin-NaCl) (○). Enzyme was also diluted with fresh pig serum and filtered through Sephadex G-25 (△). The same concentrations of enzyme were diluted in albumin and passed through Sephadex (□). The reaction time was 3 hr.

tional 1 ml of eluate. Duplicate blanks were prepared by incubating the substrate and sample separately, adding TCA to the xanthine, then 0.2 ml of sample, and centrifuging. The Dowex columns, which contained the [^{14}C]xanthine, were discarded after use.

RESULTS AND DISCUSSION

The [^{14}C]xanthine as received commercially (97% radiochemical purity) gave blanks of about 4200 cpm; therefore, purification was necessary to remove radioactive impurities which eluted from Dowex with the same elution volume as uric acid. Blanks were reduced to about 110 cpm following filtration of [^{14}C]xanthine through PVP. About 3% of the total radioactivity was contained in the initial 2.3-ml eluate, which was discarded, while 97% of the total radioactivity was found in the 1.8-ml eluate retained for use in the assay. The elution pattern does not change at pH 7.4. A better enrichment of xanthine may be obtained by taking a smaller cut, e.g., 1.5 ml, which contained 95% of the total radioactivity.

Figure 1 shows a typical standard curve obtained using buttermilk XO in albumin. Similar points were found when enzyme was diluted with fresh miniature pig serum, filtered through Sephadex G-25, and assayed.

The results were corrected for dilution due to gel filtration as well as for the XO content of the pig serum. Complete recovery of enzyme was also obtained after gel filtration of buttermilk XO in albumin. The lowest level of XO which could be accurately determined was 0.01 μU/sample, and linearity was observed to 36.4 μU/sample; duplicates agreed within $\pm 3\%$.

In studies to determine whether pig blood serum contained XO inhibitors, buttermilk XO was diluted with albumin–NaCl to 91 μU/0.1 ml. A 0.1-ml aliquot was added to 1.9 ml of pooled pig serum, and aliquots were assayed with and without Sephadex filtration. An 11-fold increase in enzyme activity was obtained by passing the serum through Sephadex before incubation. Evidently the pig serum contains low molecular weight inhibitors of XO.

Three miniature pigs, maintained on a bovine milk-free diet for 5 months had serum XO levels of 0.2–3.9 μU/ml (26 samples). Although most samples contained 0.3–0.5 μU/ml, occasionally enzyme activity above 1 μU/ml was found. Xanthine oxidase levels of 1-μU/ml serum remained stable when stored at -20°C for 3 days; however, some decrease in activity was found in samples containing 0.5 μU/ml when stored under the same conditions.

Standards containing 0.1456 to 75 μU enzyme were incubated for 1 hr and showed a linear relationship; therefore, the incubation period can be reduced to 1 hr where sufficient counts are obtained.

In order to determine whether the assay could be applicable to samples containing uricase, allantoin (Eastman) was eluted with 0.1 N HCl from a Dowex column. Total recovery of allantoin was observed (absorbance at 235 nm) at the same elution volume as uric acid.

The procedure described here, which is scaled down from that reported by Al-Khalidi *et al.* (7), uses commercially available [^{14}C]xanthine. The assay, which requires only 0.3 ml of serum, is about 20 times more sensitive than the original method and is more economical, since less than one-tenth the amount of [^{14}C]xanthine is used per sample.

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